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# BACULOVIRUSES AS GENE EXPRESSION VECTORS

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## INTRODUCTION

Although baculoviruses have been of interest for many years by virtue of their impact on pest insect populations, the recent burgeoning interest in these viruses stems from their usefulness as helper-independent viral vectors for the high-level expression of foreign genes in eukaryotes. The popularity of baculoviruses as gene expression vectors is directly related to the success that numerous academic and commercial laboratories have had in obtaining substantial quantities of biologically active products from a variety of eukaryotic genes, especially those that posed difficulties in lower eukaryotic or prokaryotic expression systems. Baculovirus-based expression is achieved rapidly using conventional virological techniques. As with other expression systems, however, the ability of the baculovirus system to provide abundant quantities of any particular foreign gene must be determined empirically, since many aspects of foreign gene expression and protein stability are not known at this time.

All currently available baculovirus expression vectors provide the advantage of a natural viral gene regulation phenomenon, namely the very late but highly abundant expression of the polyhedrin gene. This gene is expressed during occlusion, the second of two stages of the infection cycle. Each stage produces a biochemically and functionally distinct form of the virus: The extracellular budded form of the virus is produced during the first stage, and the occluded form of the virus, composed primarily of polyhedrin protein, is produced during the second stage. Knowledge of the biology and the molecular basis of the baculovirus infection process allows for a fuller understanding and appreciation of the advantages and power of the expression vector system.

This review first surveys the basic biology of baculoviruses at the organismal and molecular levels, with specific emphasis on those features relevant to the use of the viruses as expression vectors. An outline of the available baculovirus expression vectors is followed by a brief discussion of some factors that affect the quantity and biological quality of the gene products; of particular interest in this regard is the nature of posttranslational modifications in insect cells. Methods for cell propagation, recombinant virus construction, and protein production are considered elsewhere (44, 68). Prior reviews relevant to baculovirus expression vectors (37a, 44, 45, 48) and baculovirology in general (9, 12, 16, 17, 33, 67, 70) are available.

## BIOLOGY OF BACULOVIRUSES

### *Structure and Classification*

Viruses within the family Baculoviridae possess a single molecule of circular supercoiled double-stranded DNA of 80–220 kb. The DNA is packaged as

rod-shaped nucleocapsids, which acquire membrane envelopes either by budding through the plasma membrane of the cell or by a nuclear envelopment process. In the subgroup A baculoviruses known as nuclear polyhedrosis viruses (NPVs), those virions that obtain an envelope by an intranuclear envelopment process can be occluded within a paracrystalline protein matrix, forming large (1–5 $\mu$m) occlusion bodies of polyhedral morphology containing multiple virions. NPVs are further distinguished on the basis of whether they contain a single nucleocapsid (SNPV) or multiple nucleocapsids (MNPV) per envelope.

Baculoviruses have been isolated from invertebrates only. Over 400 baculoviruses have been reported in the literature, but less than 20 have been studied at the molecular level. The most intensively studied at this level is an MNPV originally isolated from *Autographa californica*, a lepidopteran noctuid (which in its adult stage is a nocturnal moth) commonly known as the alfalfa looper. The virus is known as AcMNPV or AcNPV.

Although AcMNPV has been used most extensively for gene expression vector purposes, other NPVs may also be developed as vectors based on the experience from the AcMNPV system. For example, the NPV of *Bombyx mori* (silkworm) (BmNPV) has been developed as a gene expression system (40) even though only a single gene (the highly conserved polyhedrin gene) has been identified in this virus.

## *The Two Infectious Forms of Nuclear Polyhedrosis Viruses*

The two forms of an NPV, the occluded virus (OV) and the extracellular budded virus (EV) have distinct roles in infection (reviewed in 18). OVs are involved in the horizontal transmission of virus infection from insect to insect. Insects usually acquire the disease by consuming food contaminated with OV. The paracrystalline matrix of OVs is comprised primarily of a 29-kd protein, polyhedrin. This matrix apparently has two roles in horizontal transmission: (*a*) to protect the embedded virions from inactivation during the interval of transmission between host organisms and (*b*) to effect the release of the virions at the primary site of infection, the midgut epithelial cells, by dissolving in the high-pH (10.5) environment of the midgut lumen. Systemic infection of the insect (as well as infection in cell cultures) is mediated by EVs, which bud from infected cells into the hemolymph of the insect.

The baculovirus vectors that are routinely used are based on the substitution of the polyhedrin gene with the foreign gene of interest (40, 44, 48, 49, 68). Substitution of the polyhedrin gene interferes with the production of OV but has minimal effect on the production of EV (62). Thus the polyhedrin-based substitution vectors are helper-independent vectors for gene expression in cell cultures but are defective in per os transmission from insect to insect. The lack of OV production is a visually selectable plaque phenotype and provides a

rapid means of identifying recombinant viruses (44, 62, 68). Recombinant EVs are defective only in horizontal transmission among insects via the oral infection route.

In a synchronous infection of a permissive cell line such as *Spodoptera frugiperda* (fall armyworm) IPLB-SF-21, the production of the bulk of progeny EV precedes the production of OV. The delay in OV production can be accounted for by a delay in the production of stable transcripts from occlusion-specific genes such as polyhedrin (reviewed in 14). Whereas the bulk of EV is produced within the first 18 hr postinfection (p.i.), polyadenylated transcripts from the polyhedrin gene begin to accumulate in the cytoplasm of the cells at 18–24 hr p.i. Translation products appear shortly thereafter and accumulate through 70 hr p.i. Thus in a polyhedrin-based baculovirus expression system, the production of foreign gene products is expected to have minimal impact on the ability of the recombinant virus to replicate. There should be minimal selection pressure to eliminate the foreign gene insert, providing insert stability, and it should be possible to express at least some foreign gene products that adversely affect cell viability. Although insert stability is usually observed, the latter feature has not been rigorously tested yet. A trick that might be used to facilitate the isolation of recombinant viruses expressing cytotoxic products is to utilize a host cell that supports EV production but fails to express polyhedrin [e.g. AcMNPV-mediated expression in *B. mori* cells (69)].

## MOLECULAR BIOLOGY OF AcMNPV

EVs apparently enter cells by adsorptive endocytosis (73). The rod-shaped nucleocapsids enter the nucleus, where the nucleoprotein core is released from the capsid (71). The core of AcMNPV is comprised of the 128-kb supercoiled DNA associated with a protamine-like, arginine-rich 6.9-kd protein (74). Although very early events are difficult to study, some data suggest that the viral DNA may be released from this core and assume a nucleosome-like structure using host-encoded histones. Late in infection, the viral DNA, but not the host DNA, assumes a unique nucleoprotein structure as observed by micrococcal nuclease protection experiments (75). At least three phases of AcMNPV gene expression can be distinguished in AcMNPV-infected *S. frugiperda* cells: (*a*) an early phase, (*b*) a late phase, and (*c*) a very late or occlusion-specific phase.

### *Early Phase of Gene Expression*

During the early phase of gene expression (from 0 to approximately 6 hr p.i.), which precedes viral DNA replication, a number of early genes that are distributed throughout the AcMNPV genome are expressed (reviewed in 14). There may be two classes of early gene transcription: immediate early and

delayed early. Transient expression assays suggest that the product of the IE-1 gene can act as a *trans*-acting transcriptional activator of other early genes (22). It remains to be demonstrated genetically whether IE-1 has such a role in early gene expression or whether it is a component of the virion. The effects of IE-1 activation in transient assays are dramatic; thousandfold increases in gene expression are observed when a "homologous region" sequence is present in *cis* with the reporter gene (23). There are six short (400–900 bp) homologous regions (hr1, hr2, hr3, hr4l, hr4r, and hr5) interspersed through the genome of AcNPV (13, 21); the nucleotide sequences of these regions reveal the reiteration of an approximately 60-bp sequence encompassing a highly conserved 26-bp imperfect palindrome, the center of which is an *Eco*RI site (21, 37). These sequences, in conjunction with IE-1, resemble enhancers (21, 23) and, by analogy with other DNA viruses, may serve as origins of viral DNA replication.

### *Late Phase of Gene Expression*

The late phase of AcMNPV replication, extending from approximately 6 through 18 hr p.i., is characterized by extensive replication of viral DNA and the formation of progeny EV. This phase encompasses the production of structural proteins of nucleocapsids, including the major capsid and the core proteins, which have been sequenced recently (74; S. M. Thiem & L. K. Miller, unpublished data). Another abundant late protein is a 64-kd glycoprotein that is found in the envelope of EVs, but not OVs, and that has a role in receptor-mediated entry of EV into cells (73).

### *Very Late Phase of Gene Expression*

The final phase of gene expression, from approximately 20 through 72 hr p.i., is referred to as the occlusion phase or very late phase of gene expression. This phase is characterized by abundant polyhedrin synthesis and the formation of OV within the nucleus. By 70 hr p.i., wild-type AcMNPV produces an average of 70 OVs per nucleus; each OV is approximately 2 $\mu$m in diameter and contains numerous enveloped nucleocapsids. Polyhedrin becomes the most predominant protein of the cell, composing 25–50% of the total stainable protein of the cell by 70 hr p.i. The expression of at least one other protein of AcMNPV, the abundant p10 protein, is regulated in a similar fashion to that of polyhedrin (reviewed in 13, 14). Although p10 is not essential for the formation of refractive occlusions, it is thought to be involved in the maturation of OVs and possibly in the cytoskeletal structure (P. Faulkner & G. Rohrmann, personal communication).

### *Late Promoters*

The two late phases of gene transcription are apparently mediated by a virus-induced $\alpha$-amanitin–resistant RNA polymerase (15, 20). It is not yet

known whether the virus encodes any portion of this induced polymerase. The polymerase probably recognizes, directly or indirectly, a consensus sequence that serves as the initiation site for RNA synthesis for all known late and occlusion phase–specific genes characterized to date (61). Based on the sequences of the 5' start sites of eight different major late RNAs of AcMNPV [polyhedrin, p10, the 6.9-kd core protein, the 39-kd late start site, two transcripts of the major capsid protein (S. M. Thiem & L. K. Miller, unpublished data), and the long terminal repeats (LTRs) of AcMNPV-borne transposable element D], the following consensus sequence has emerged: a-a/t-a/t-y-a/t-a/t-A-T-A*-A-G-a-a-a/t-t-a/t-a/y-a/t-a/t-t. The capital letters ATAAG represent invariant nucleotides. The bases represented by small letters are usually found in seven of eight sequences (y represents a pyrimidine). The asterisk notes the position of the 5' start site that my laboratory has determined for six of the eight abundant late transcripts (polyhedrin, the 6.9-kd core protein, the capsid transcripts, and the LTRs of transposable element D).

The effects of deletions in the region upstream of the polyhedrin coding region have suggested that this consensus sequence and additional downstream sequences are intimately, and possibly exclusively, involved in the abundant expression of this gene (25, 43), although distant enhancers such as the homologous regions may have a role. In the most extensive study reported to date, a series of AcMNPV expression vectors carrying the infuenza virus hemagglutinin (HA) gene in lieu of the polyhedrin gene was constructed such that the lengths of the RNA leaders differed (43). Recombinants lacking 7, 11, 14, 16, 27, 31, 46, 51, and 58 bases immediately upstream of the original polyhedrin ATG were assayed for HA activity. A significant reduction in HA activity was observed between −16 and −27. The HA assays may not have been sensitive enough to discern subtle differences between −1 and −27. SDS–polyacrylamide gel analysis (43) provided evidence that the region from −1 to −8 is essential in obtaining maximum levels of at least one foreign gene product.

Sequences upstream of −70 contribute minimally if at all to polyhedrin expression. Large insertions of nonviral DNA at the *Eco*RV site at −92 suggested that the region upstream, with the possible exception of distant enhancers, is unessential for high-level expression (56a). Successive deletions from the *Eco*RV site toward the ATG have suggested that the region from approximately −69 to −92 has little or no effect on polyhedrin promoter-driven gene expression (56a). These results are consistent with experiments in which the 92 bp upstream of the polyhedrin ATG, a chloramphenicol acetyltransferase (CAT) gene fusion, and the polyadenylation site of the transferase gene were excised from a pEV55-based plasmid vector (Figure 1) and placed in opposite orientation within the plasmid vector (C. Rankin, B.-G. Ooi & L.

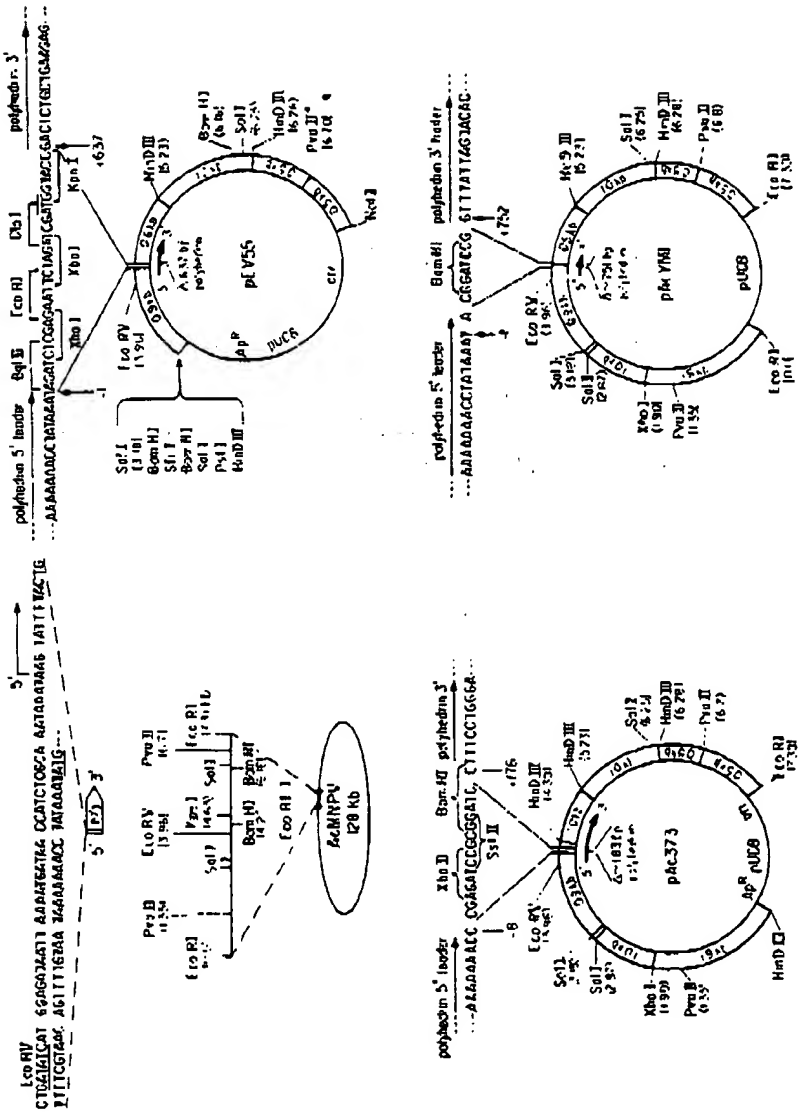
K. Miller, manuscript in preparation). Transient expression of plasmids carrying the CAT gene in reverse orientation resulted in higher levels of CAT activity than is observed for the normal orientation following cotransfection of recombinant plasmids with wild-type viral DNA.

We have recently constructed a series of pEV55-based, CAT-containing plasmids with linker-scanning mutations through the −1 to −92 upstream polyhedrin promoter/leader region (C. Rankin, B.-G. Ooi & L. K. Miller, manuscript in preparation). Transient expression assays of CAT activity following cotransfection of the plasmids with wild-type viral DNA indicated that linker replacement in the late promoter conserved consensus sequence (see above) inactivates the promoter. Virtually no effect on CAT expression was observed for linker replacement upstream of −60. Severalfold decreases were observed for some linker replacements in the −1 to −40 region. Initial experiments with recombinant viruses carrying these linker-scanning mutations confirm the results observed by transient expression assays.

The region from −1 to −60, encompassing the entire 50-base leader region, appears to be sufficient, in the context of the viral genome, for abundant polyhedrin expression. As noted below, vectors that lack any portion of the leader sequence may not provide optimal levels of foreign gene expression. It is not yet known how addition of nucleotides to the leader sequence may influence transcription and/or translation of foreign genes, although expression of genes with long leaders has been reported. It is recommended that such additional nucleotides be kept to a minimum during vector construction. Since the polyhedrin leader is A+T rich, the inclusion of G+C-rich sequences in the leader should be avoided, if possible.

To date, there is no convincing evidence to suggest that fusions to polyhedrin coding sequences are more highly transcribed than genes inserted at +1 of the polyhedrin coding region. Such fusions are unessential for the abundant expression of at least some genes: expression of the lymphocytic choriomeningitis virus (LCMV) N gene, when the gene is inserted at +1 and employs its own ATG, reaches levels representing 50% of the total stainable protein of the infected cell (43). Much remains to be learned about RNA stability and translational efficiency in baculovirus-infected cells. We do know that not all genes will be expressed efficiently even if inserted at the +1 position.

Signal sequences or polyhedrin coding region fusions may be useful in providing stability to otherwise unstable proteins by altering cellular location or increasing resistance to proteolysis. In a recent study, expression of a gene encoding a 36–amino acid scorpion toxin was not detectable even though the synthetic gene was inserted so that its ATG was six nucleotides downstream from the −1 position (L. F. Carbonell, M. R. Hodge, M. D. Tomalski & L. K. Miller, manuscript submitted). However, attachment of a signal sequence



in-frame with the gene resulted in expression that was detectable by [$^{35}$S]methionine labeling. Fusion of the 36-codon gene to the N-terminal 58 codons of polyhedrin allowed synthesis that was detectable by Coomassie blue staining. Although levels of foreign gene transcripts for all three recombinants were equivalent to those of polyhedrin RNA in wild-type infections, foreign protein levels were far lower than those of polyhedrin. Thus expression of this peptide is apparently limited at the level of translational efficiency and/or protein stability.

A codon bias in the highly expressed late baculovirus proteins has been noted (61). It is unlikely that this codon bias seriously affects the translational efficiency of most genes because very high levels of $\beta$-galactosidase gene expression have been observed (55), and this large gene contains many codons that are underrepresented in late, highly expressed baculovirus genes. Attention should be given to the context of the initiating ATG of the foreign gene. The initiating ATG of late, highly expressed baculovirus genes is usually AANATG.

## *Gene and Transcript Organization*

The mapping of the transcripts of AcMNPV and the sequencing of approximately one third of the AcMNPV DNA genome (reviewed in 13, 14; recently extended in 24, 37, 52, 74; L. K. Miller, M. D. Tomalski, J.-G. Wu, S. M. Thiem & D. R. O'Reilly, unpublished data) have revealed the following. (*a*) Coding regions are arranged in linear nonoverlapping units, which are usually separated by A+T-rich regions. (*b*) Tandem or bidirectional arrangements of open reading frames are observed. (*c*) The transcriptional initiation signals and polyadenylation sites often overlap in the A+T-rich regions. (*d*) Transcriptional motifs include multiple overlapping RNAs with common 5' or 3' termini; it is not uncommon for one open reading frame to be transcribed in both directions into early- and late-abundance polyadenylated RNAs. (*e*) Early and late transcripts often overlap, perhaps for regulatory purposes. (*f*)

*Figure 1* The design of AcMNPV transplacement plasmids. *Upper left:* Location of the polyhedrin gene of AcMNPV within the 7.3-kb *Eco*RI-I fragment of the 128-kb genome (72). The position and direction of the polyhedrin gene within this *Eco*RI fragment are shown above. The sequence of the promoter/leader region beginning at the *Eco*RV site at −92 is provided. The start site for polyhedrin gene transcription is indicated (5' arrow). *Upper right, lower left, lower right:* AcMNPV transplacement plasmids pEV55 (44), pAc373 (64, 68), and pAcYM1 (43). All are very similar in their basic design. Regions of the *Eco*RI-I fragment of AcMNPV are shown as double-lined plasmid segments. All three plasmids have portions of the polyhedrin gene deleted; the size of the deletion is indicated within the circle. In place of those deletions, multicloning sites have been inserted. The sequence of the sites and their positions relative to the polyhedrin leader are shown. The numbers (e.g. −1, +637) are relative to the original polyhedrin ATG (+1, +2, +3). Key restriction sites in the plasmids are indicated. All plasmids are pUC-based and confer resistance to ampicillin.

Early, late, and very late genes are interspersed in the genome; no pattern for gene arrangement has been discerned yet. (*g*) All characterized coding regions, including early, late, and very late genes, lack introns; splicing of natural viral transcripts has not been observed to date.

It is recommended that all foreign genes to be expressed abundantly in the baculovirus expression system be inserted without introns (e.g. as cDNAs). Introns of SV40 tumor antigens were reported to be removed from RNA transcripts in recombinant-infected cells (31). However, large T-antigen expression, which is dependent on correct splicing, was not observed (31). Small t antigen was expressed, but its synthesis is not dependent on correct splicing. We have observed excellent synthesis of large T antigen from a recombinant carrying an intronless version of this SV40 gene (D. R. O'Reilly & L. K. Miller, manuscript submitted). Although it is possible that foreign genes with introns may be expressed in baculovirus-infected cells, it is unlikely that introns will augment expression and probable that they will inhibit expression.

## TRANSPLACEMENT PLASMIDS FOR RECOMBINANT VIRUS CONSTRUCTION

Owing to the large size of baculovirus genomes, most recombinant virus construction relies on in vivo recombination to replace a viral allele with the gene of interest (40, 46, 49, 55, 62, 64). Transplacement plasmids contain the site for foreign gene insertion as well as flanking viral sequences, which provide homologous sequences for recombination. Cotransfection of cells with viral and recombinant plasmid DNAs allows cell-mediated allelic replacement of the target viral gene with the plasmid-borne foreign gene.

### *Vectors for High-Level Expression of a Single Gene*

The polyhedrin promoter and its flanking sequences are usually employed for allelic replacement into the AcMNPV genome because the polyhedrin promoter/leader region provides abundant transcription and because replacement of the polyhedrin gene with a foreign gene allows visual selection of recombinants on the basis of their occlusion-negative (occ$^-$) phenotype. Three similar AcMNPV transplacement plasmids are schematically presented in Figure 1; analogous vectors are available for BmNPV (25, 50). All of these vectors have linkers with one or more unique restriction sites in place of all or part of the polyhedrin coding region. They also have sufficient flanking viral sequences for efficient recombination into the viral genome. The pAc373 vector, in contrast to pEV55 or pAcYM1, has a linker positioned at $-8$ rather than $-1$ relative to the original polyhedrin ATG (+1, +2, +3). Since these

eight nucleotides are implicated as important components for optimal expression (43; see above), optimum levels of expression of some genes may not be achieved with this vector.

The three vectors differ in the nature of the linker. In addition to unique sites that can be used to position genes for expression using their own ATG, pEV55 has a terminal *Kpn*I site that can be filled in with a polymerase to provide a blunt end containing a translational initiation codon (ATG) to allow expression of cDNAs lacking their own initiation signal (37a, 44). Other vector systems are available to provide in-frame fusions to varying lengths of the polyhedrin N-terminus (37a, 68). Although it is not clear that such fusions provide additional advantage to a +1 vector system, they are useful for expression of genes lacking their own ATG and may provide protein stability (see above). In expressing a synthetic gene using BmNPV, a polyhedrin fusion protein was produced abundantly, and the polypeptide was subsequently liberated by cyanogen bromide cleavage; biological activity of this product was not reported (41).

The variation in the length of the deletion of polyhedrin coding sequences (Figure 1) presumably has no effect on foreign gene expression; the largest deletion (13 nucleotides of the trailer region in pAcYM1) can provide levels of expression equivalent to those of polyhedrin in the case of at least one gene (43). All the vectors include the polyadenylation signal of polyhedrin. It is not known whether this is an important feature; foreign genes containing their own polyadenylation signals can be efficiently expressed.

### *Expression of Two or More Foreign Genes*

For many applications, it is of interest to express two or more foreign genes simultaneously in the same cells (e.g. multisubunit proteins). One simple way to achieve this goal is to construct separate recombinant viruses (e.g. using the vectors described above) so that each virus expresses one of the protein subunits. The viruses can be mixed in appropriate proportion and used for cell inoculation. As long as the multiplicity of infection for each virus is at least 5 plaque-forming units (PFU) per cell, over 95% of the cells will be infected with at least one of each of the viruses, which ensures coexpression of the genes in the same cell. This coinfection technique was applied in a study of complex formation between two subunits of the influenza virus polymerase (65) and in a study demonstrating complex formation between SV40 T antigen and mouse p53 protein (D. R. O'Reilly & L. K. Miller, manuscript submitted).

Transplacement vectors specifically designed to allow high-level coexpression of two or more genes are in the development stage. One approach has been to insert a foreign gene (the LCMV N gene), under polyhedrin promoter control, upstream of and in opposite orientation to the polyhedrin gene (10).

The insertion site utilized was the *Eco*RV site, −92 bp upstream of the polyhedrin ATG (43). The occ$^+$ recombinant thus has two polyhedrin promoters in opposite orientations, one driving polyhedrin expression and one driving LCMV N expression. The next step would be to replace the polyhedrin gene with a second foreign gene of interest, presumably by selecting for a polyhedrin occ$^-$ phenotype. One important question regarding this system, which remains to be tested by Southern blot analysis, is whether the duplication of the polyhedrin promoter leads to recombinant virus instability. Since only approximately 60 nucleotides of the polyhedrin promoter appear to be necessary for high-level gene expression (see above), it is probable that very convenient systems for expression can be developed that will minimize promoter homology. Other dual expression systems may be developed to exploit the very late p10 promoter. Additional methods for identifying recombinant viruses are available besides selection for occlusion body phenotype; both plaque hybridization to DNA probes and β-galactosidase (*lacZ*) gene marker systems can be used (44, 68).

Another system for dual gene expression utilized a polyhedrin/*lacZ* fusion as a marker for recombinant virus selection (6), while the CAT gene, under the control of the Rous sarcoma virus long terminal repeat (RSV-LTR), was inserted downstream of the *lacZ* gene. The occ$^-$, blue-plaque recombinant virus expressed β-galactosidase and CAT in a temporally distinct fashion. The transplacement plasmid may be used for inserting genes under control of their own promoter. A gene under polyhedrin or p10 promoter control could be inserted into this vector, although the stability of a construct containing a duplicated promoter must be determined as noted above. Replacement of the polyhedrin/*lacZ* fusion gene with a foreign gene can then be achieved with an appropriately constructed transplacement plasmid, and recombinant viruses can be selected as non-blue-plaque viruses in the presence of a chromogenic indicator.

## *Baculovirus Pesticide Applications*

Foreign gene expression in baculoviruses is also of interest in the development of more effective baculovirus pesticides (6, 7). Most applications of baculovirus expression vectors have been aimed at producing foreign proteins as abundantly as possible and have therefore utilized polyhedrin or related promoters to drive gene expression. For pesticide applications it may be preferable to utilize an early promoter to drive the expression of the foreign gene (i.e. an insect-specific neurotoxin) and to retain polyhedrin expression so that OVs are produced (6, 7). A recombinant AcMNPV containing a marker oligonucleotide was recently field-tested in the United Kingdom. Virus persistence and spread in the environment were monitored with a view toward future development and release of genetically improved pesticides (3).

## SELECTION AND MAINTENANCE OF VIRUS VECTORS AND HOSTS

The nature of the virus and the host used for expression deserve consideration. It is beyond the scope of this review to deal with these aspects in depth, but virus stability and hosts are briefly considered.

### *Virus Stability*

Baculoviruses, like most other animal viruses, undergo genomic alteration upon serial passage in cell culture (reviewed in 47; see also 35). The most obvious manifestation of such changes is a general decline in the ability of NPVs to produce OVs after serial passage of EVs by routes that eliminate the need for OVs (i.e. cell culture or hemolymph injection of larvae). Although for historical reasons this phenomenon has been documented most extensively for passage of AcMNPV and its closely related variant *Trichoplusia ni* MNPV (TnMNPV) in *T. ni*, the same phenomenon probably also occurs for other viruses and other hosts. Serially passaged EV stocks usually exhibit the few-polyhedra (FP) phenotype, characterized by the production of fewer than 10 polyhedra per infected cell. FP mutants eventually predominate in serially passaged stocks owing to a selective growth advantage in cell culture; at least some FP viruses produce a higher titer of EV than the wild-type viruses (reviewed in 47, 57, 58). The FP phenotype of at least some FP mutants is apparently due to a defect in the nuclear envelopment of nucleocapsids, which is a prerequisite to occlusion. The lack of nuclear envelopment provides a rationale for the overproduction of EVs and the selective growth advantage of the FP phenotype in serial passages that avoid the oral route of infection. Whether there is less polyhedrin synthesis in FP than in wild-type infected cells is somewhat controversial; FP mutants do make high, but possibly not optimum, levels of polyhedrin.

Generation of FP virus might have the following impacts on the use of the virus for expression purposes. (*a*) If viral DNA from serially passaged virus stocks is used in the initial cotransfection for recombinant DNA selection, the visual selection for the occ$^-$ phenotype will be obscured by the FP phenotype. (*b*) Amplification of a recombinant virus by long-term serial passage can result in a decline in expression. Both problems can be minimized, if not solved, by careful attention to the quality of the virus stock. For wild-type stocks, a wild-type plaque can be visually selected by its many-polyhedra (MP) phenotype. The virus can be amplified into large (500 ml) stocks within two to three passages; most of the second- or third-passage stock can be reserved for generation of larger third-, fourth-, or fifth-passage stocks. Stocks can be monitored by plaque assay for the integrity of the wild-type phenotype. For recombinant virus stocks whose MP phenotype cannot be checked easily, the best approach is again to limit the number of serial

passages of the virus to six or less and to initiate new stocks from early-passage stocks of the characterized recombinant.

## *Production in Cell Culture Versus Insect Larvae*

Although most expression work has thus far been conducted in cell culture, it is likely that many laboratories will desire large quantities of product and will begin to explore avenues of expression that will minimize costs of production. One possibility that has been suggested is production in insect larvae (40, 50). The decision of whether to produce a foreign protein in larvae rather than in cell culture may depend on the amount of product required, the ease of purification from larval carcasses, the purity of the product necessary, the relative convenience of the two modes of production, and the comparative costs. The questions of cost, convenience, product stability, and purity may need to be balanced for each product and may even depend on the application of each product.

The use of insect larvae for production purposes has been promoted because levels of protein products in hemolymph exceed those in cell culture (40, 50). In two of three cases (40, 41, 50), hemolymph contained a tenfold higher concentration of product (units or mg $ml^{-1}$) than cell culture media; approximately 0.5 ml of hemolymph are obtained per silkworm, whereas 5 ml of media are used per $1.5 \times 10^6$ cells. In one of those cases, 3.6 mg of a polyhedrin/insulin-like growth factor II (IGF-II) fusion protein were recovered per larva, and 0.3 mg protein were produced per milliliter of culture medium (41). At these levels it would be necessary either to inject 300 larvae or to infect 3 liters of cells to obtain 1 g of purified product. In the third case, that of mouse interleukin-3 (IL-3) expression (50), there was a significantly higher (i.e. approximately 500 fold) level of biologically active protein in hemolymph than in cell culture media. It is not clear why IL-3 expression differs from that of the other two proteins studied; it is possible that some proteins are more susceptible to proteolysis in cell culture media (which usually contain fetal bovine serum) than in insect hemolymph. In the case of IL-3, it would appear that production in larvae is preferable. IL-3 is secreted into the hemolymph, and purification from hemolymph is easily achieved. Whether collection of hemolymph can be performed on a large-scale basis remains to be demonstrated; if not, purification from entire larvae would be necessary, as for proteins that are not secreted into the hemolymph. This may prove to be more challenging.

Marumoto et al (41) advocate expression in silkmoths because of their large size, the long history of sericulture, and computerized mass-rearing systems. The difference in larval size may be a consideration to the bench scientist who is considering injecting EV into hemocoels and collecting hemolymph. *T. ni* larvae, which support AcMNPV vectors, are approximately one third the size of *B. mori* larvae. Although *T. ni* have not been reared by humans for 3000

years, laboratory strains are conveniently reared in large numbers and are routinely available for research purposes. Rearing of silkmoths is considered expensive by those in the United States who have experience in mass rearing of insects for viral pesticide production (28; C. M. Ignoffo, personal communication). Such economic considerations, however, are primarily within the realm of industrial-scale production.

The development of vectors that allow dual expression of both polyhedrin and foreign genes so that OV are produced (see above) may make the use of insect larvae for large-scale protein production more convenient; an $occ^+$ virus can be introduced as a contaminant of the food supply, while $occ^-$ viruses must be individually injected into the hemocoel. Occlusion of $occ^-$ recombinant viruses can currently be achieved by coinfection of cultured cells with wild-type and recombinant virus with a multiplicity of infection (MOI) of 5 or more PFU per cell; thus both genes are expressed in the same cell, and the polyhedrin, supplied by the wild type, can occlude the recombinant virions. It remains to be determined whether the coexpression of polyhedrin interferes with the expression and/or purification of the foreign protein. Possible difficulties of purifying protein from larvae remain.

Another avenue currently being explored by some laboratories is reduction in the cost of insect cell culture media. Most media for the culture of lepidopteran cells in the laboratory are supplemented with fetal bovine serum. For a number of years government and industrial laboratories have undertaken to reduce the cost of media for mass production of baculoviruses for pesticide purposes; methods are often retained as proprietary. Much work remains to be done, but rapid progress is likely based on experience from mammalian cell culture work.

## *Host Range of AcMNPV*

AcMNPV has a relatively wide host range for a baculovirus; it is known to infect productively at least 33 different species within 10 different families of Lepidoptera (19). It is not known to infect productively any species outside this order. The IPLB-SF-21 cell line of *S. frugiperda* is usually used for expression work because it performs excellently in both monolayer and suspension culture.

Using a dual marker gene recombinant of AcMNPV, it was possible to demonstrate that AcMNPV enters dipteran cells (e.g. *Drosophila* and *Aedes*) and efficiently expresses the CAT gene under the control of the RSV-LTR, which behaves at least in part as an early promoter in permissive *S. frugiperda* cells (6). Late genes, however, are not expressed in these recombinant virus–infected dipteran cells, and the infection is not productive (6, 60). Expression of the RSV-LTR CAT gene is not observed in recombinant AcMNPV-infected mammalian cells, which indicates that there is a block in the ability of AcMNPV to deliver its DNA genome to the mammalian nucleus

(7). Virus entry into the cytoplasm of mammalian cells has been confirmed by recombinant virus studies (7), but the efficiency of entrance appears to be low, and the progress of the virus is apparently blocked, perhaps in the lysosomes, prior to nuclear entry or uncoating. These observations support the view that baculovirus vector systems are relatively safe with respect to mammals.

## POSTTRANSLATIONAL MODIFICATION AND BIOLOGICAL ACTIVITY OF BACULOVIRUS-PRODUCED PROTEINS

A wide variety of genes have been expressed in baculovirus expression vectors. These include genes for (*a*) immune regulatory proteins such as human alpha interferon (25, 39, 40), beta interferon (64), IL-2 (63), and IL-3 (50); (*b*) DNA-binding proteins such as *Drosophila* Kruppel gene product (53), *Neurospora* qa-1f activator (2), human T-cell leukemia virus type 1 (HTLV-1) p40$^{x}$ transactivator protein (30), c-*myc* (51), and the large T antigens of SV40 and polyomavirus (59; D. R. O'Reilly & L. K. Miller, manuscript in preparation); (*c*) virus structural proteins such as LCMV N and G proteins (42, 43), influenza hemagglutinin proteins (36, 56), rotavirus VP6 (11), bluetongue virus VP2 (29), human immunodeficiency virus (HIV) *gag* (38) and *env* proteins (27), phlebovirus N protein (54), parainfluenza virus HA neuraminidase (HN) (8), and hepatitis B virus surface antigen (32); and (*d*) other proteins such as SV40 small t antigen (31), influenza virus polymerase genes (65), IGF-II (41), phlebovirus $NS_s$ protein (54), β-galactosidase (55), and CAT (6). Expression of many other genes is under way in numerous academic and industrial laboratories.

Many researchers are using eukaryotic expression vectors, rather than prokaryotic or lower eukaryotic vectors, to optimize the likelihood that the product will be biologically active. Many factors may affect the biological activity of a eukaryotic protein, including posttranslational modifications (e.g. glycosylation, proteolysis, phosphorylation, ADP-ribosylation, acylation, sulfation) and the tertiary structure (e.g. disulfide bond formation) or quaternary structure (e.g. oligomerization or complex formation). Since numerous genes that are being expressed with baculovirus vectors are of mammalian origin, the question of how posttranslational modification and cellular localization in baculovirus-infected insect cells will compare with those observed in mammalian cells becomes particularly important. Several of these factors are considered below.

### *Proteolysis, Including Signal Sequence Cleavage*

Insect cells apparently recognize and cleave mammalian signal sequences that direct proteins to the endoplasmic reticulum (ER). The signals of human alpha

and beta interferons (40, 64) and interleukins 2 and 3 (50, 63) are all recognized and cleaved in insect cells as in mammalian cells. It thus seems likely that most, if not all, proteins with appropriate mammalian signals will be transported to the ER and cleaved in a normal fashion. Whether similar signal recognition will occur in lysosomal or mitochondrial protein transport remains to be determined.

Certain other specific proteolytic cleavages have also been observed for other baculovirus-produced proteins. The HA protein of at least one influenza virus was cleaved, albeit slowly, in baculovirus-infected cells (36); in another report cleavage was not observed (56). The HA cleavage is a biologically important one associated with fusion activity and viral pathogenicity; the differences in HA susceptibility to cleavage by a host cell appear to be correlated with pathogenicity of the virus. The HIV envelope protein is also cleaved slowly, but apparently accurately (27). Both HA and env cleavage involve an Arg-Lys rich recognition site. The HIV *gag* gene product is also proteolytically cleaved in baculovirus-infected cells; one cleavage is gag-protease related, but host-mediated cleavage is apparently responsible for a second proteolytic event (38).

## *Glycosylation*

Protein glycosylation is thought to have a variety of different roles, including protein stabilization, cellular interaction, and intracellular protein localization; posttranslational modifications of this sort may be important in biological activity of some but not necessarily all proteins. The most common and most thoroughly characterized type of mammalian glycosylation is *N*-linked glycosylation in the ER, where a common oligosaccharide is attached to asparagine in a reaction that is mediated by a phospholipid carrier. *N*-Linked glycosylation is sensitive to inhibition by tunicamycin in both mammalian cells and baculovirus-infected insect cells (34, 66).

From one careful comparative study, it appears that the sites that are targeted for glycosylation in insect cells are the same as those of mammalian cells (26). However, this study also demonstrated that the nature of the oligosaccharide at these sites differs in insect- and mammal-derived protein. Mammalian cells extensively modify the core oligosaccharide in terminal glycosylation events involving the transfer of glucosamine-galactose and sialic acid residues to form complex oligosaccharides. Insect cells appear to lack galactose and sialic acid transferases (4, 5) and trim the oligosaccharide to a central core of $GlcNAc_2Man_3$ (26). Whether this is generally the case remains to be demonstrated.

A variety of baculovirus-produced mammalian gene products are known to be glycosylated, e.g. influenza HA (36), parainfluenza HN (8), beta interferon (64), HIV *env* protein (27), and IL-3 (50). Glycosylation is sensitive to tunicamycin inhibition and can be detected with a [$^3$H]mannose label (61).

Differences in the nature of the glycosylation were suggested by differences in the electrophoretic mobility of HA and IL-3 (36, 50), but no difference in biological activity was observed. No glycosylation of IL-2 in insect cells was observed (63). However, glycosylation patterns in mammalian cells also differ; 40% of the IL-2 from one human T-cell line was not glycosylated.

It is likely that differences between insect-mediated glycosylation and mammalian glycosylation will be observed. Whether such differences influence biological activity of the proteins in a positive, neutral, or negative fashion remains to be determined for individual expression products. Indeed, the baculovirus expression system may be ideal for addressing some of the questions regarding the role of glycosylation in protein structure and function.

### *Phosphorylation*

Three nuclear proteins are reported to be phosphorylated in baculovirus-infected insect cells: the Kruppel gene product of *Drosophila* (53), the HTLV-1 $p40^{x}$ protein (30), and *c-myc* (51). Thus far, the only data reported indicate that some phosphorylation occurs, but it is not known whether phosphorylation occurs in a position identical to that in proteins isolated from the natural host cell. One report (31) has alluded to unpublished data indicating that the phosphorylation patterns are "virtually identical." More information is necessary on the efficiency and accuracy of insect phosphorylation.

### *Oligomerization and Complex Formation*

It is likely that baculovirus-infected cells will provide an adequate in vivo setting for complex formation for most proteins as long as all required factors for complex formation are present and their cellular location is appropriate for complex formation to occur. The rotavirus VP6 protein exhibits disulfide bond–dependent oligomerization; this virus structural protein also shows evidence of morphological unit assembly (11). In vivo complex formation between two of the three subunits of the influenza virus polymerase has been reported (65). However, the failure of the third polymerase subunit to form a complex may reflect either the lack of a necessary factor for successful complex formation in this system or simply the inability of insect cells to provide an adequate environment or modification necessary for complex formation. The baculovirus expression system might prove useful for exploring factors or modifications necessary for such complex formation.

### *Cellular Location*

From the cellular localization studies undertaken thus far, it appears that mammalian proteins segregate in insect cells as they would in mammalian cells. The *c-myc* gene product, for example, is found in the nucleus of

baculovirus-infected cells (51); it remains to be formally demonstrated that insect cells recognize the same nuclear transfer signals as mammalian cells. The influenza virus HA and parainfluenza HN proteins are located at the cell surface (8, 36, 56) as expected for these membrane proteins. The LCMV N gene product forms cytoplasmic inclusions, which suggests that this viral structural protein forms natural aggregates, whereas a glycoprotein of LCMV, GPC, is directed to the cell surface (42, 43); hyperexpression of GPC results in a highly vacuolated cytoplasm and dense ER (43). It remains to be determined whether this is a general cellular response to hyperexpression of glycoproteins. Efficient secretion of hepatitis B virus S-antigen, human alpha interferon, beta interferon, IL-2, and IL-3 are observed. The ability to secrete IL-3 in soluble form is considered an advantage over prokaryotic expression and a key to obtaining a product with high specific activity (50). Some proteins that are not normally secreted may also be observed in the media of infected cells; for example, rotavirus VP6 is observed in media as well as in cells (11). Whether this extracellular location is due to premature cell lysis or facile entry into the ER remains to be determined.

### *Biological Activity*

The interleukins and interferons expressed with baculovirus vectors are biologically active (40, 50, 63, 64). The most extensive study of this activity indicated that IL-3 has specific activity equivalent to that produced in mammalian cells (50); the specific activity of IL-2 is also similar to that of mammal-derived material (63). Baculovirus-produced HTLV-1 $p40^x$ exhibited transactivation of an HTLV-1 LTR-promoted globin gene in insect cells (30). The *Neurospora crassa* qa-1f activator and polyomavirus T antigen exhibited specific DNA-binding properties in vitro (2, 59). Baculovirus-derived influenza HA protein has full biological activity despite a difference in the nature of the glycosylation (36). This HA protein elicits neutralizing and protective antibodies. For viral proteins, it is important that major epitopes be displayed if they are to be used for diagnostic or vaccine purposes. Thus far baculovirus-derived antibodies raised against simian rotavirus VP6, parainfluenza virus HN, bluetongue virus VP2, HIV *gag*, and HIV *env* proteins have been useful as diagnostic reagents. In appropriate cases they have provided experimental animals with protection from virus challenge. One study noted that antisera raised to the baculovirus-derived antigen did not react with mammalian cell proteins (11). In another study a low cutoff value and a high signal-to-background ratio were obtained (27). Baculovirus-derived proteins may thus require less purification prior to being used as antigens. Baculovirus-derived HIV *env* protein is being tested in humans as a vaccine to protect against AIDS. The vaccine was developed by MicroGeneSys, Inc (West Haven, Connecticut).

## SUMMARY AND FUTURE PROSPECTS

Baculoviruses are useful vectors for the high-level expression of genes in eukaryotes. The success in constructing vectors that produce biologically active protein products using relatively easy and rapid technology makes this vector system a popular one from a research perspective. Initial studies have shown that posttranslational modifications such as signal cleavage and glycosylation are similar in insect and mammalian cells, but glycosylation apparently varies in detail. Although it is currently possible to coexpress two or more genes in the same cell, new expression vectors with additional capabilities including high-level multigene expression are being developed. Scaleup of production will benefit from additional attention to the costs of media and the improved design of vectors for expression in insect larvae. It will be fascinating to determine the mechanism(s) that regulate the expression of baculovirus genes.

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